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A delayed and sustained rise of cytosolic calcium is elicited by oxidized LDL in cultured bovine aortic endothelial cells

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Bovine aortic endothelial cells (BAEC) pulsed for 5 h with mildly oxidized low density lipoproteins (LDL), exhibited a broad, sustained and high peak of $[Ca^{2+}]_i$ occurring several hours after the end of the pulse and reaching very high $[Ca^{2+}]_i$ values (around 2500–3000 nmol/l) and a concomitant drop of cytosolic pH (around 0.2–0.3 pH units) without any loss of cell viability. When BAEC were continuously pulsed with oxidized LDL, the peak of $[Ca^{2+}]_i$ was more sustained than in short pulse experiments and was associated with irreversible morphological changes usually associated with cytotoxic events (blebbing) and with a marked loss of viability. The potential involvement of these biochemical and morphological changes in atherogenesis are discussed.

Calcium; Cytotoxicity; Oxidized LDL; Endothelial cell; Atherosclerosis; Quin-2/AM; Fluo-3/AM

1. INTRODUCTION

Low density lipoproteins (LDL) play a major physiological role in delivering cholesterol to peripheral cells, but are also involved in atherogenesis [1–5]. LDL can undergo postsecretory modifications resulting from oxidation induced by cells and/or by transition metal ions which induce apoB alterations and lipid peroxidation [2,5–7]. Recent studies suggest that oxidized LDL are involved in the genesis of atherosclerotic lesions [3–7]: (i) apoB alterations result in reduced LDL uptake through the apoB/E-receptor mediated pathway and enhanced uptake through the scavenger-receptor pathway of macrophages, leading to 'foam' cell formation (characteristic of the early lesions of atheroma) [1,5]; (ii) lipid peroxidation of LDL [3,6,7] results in a cytotoxic effect towards cultured cells [8–11]. Such cytotoxic events seem to be involved in the progression of the vascular lesions of atherosclerosis, i.e. genesis of necrotic lesions of the atheroma and defect of the endothelial cell lining [5,12,13]. In order to further investigate the mechanism of this 'cytotoxic' effect, we have recently developed a new experimental model system consisting of LDL irradiated by UV-C [14–16] which promote lipid peroxidation without detectable change in apoB-100 [14,15]. UV-treated LDL are taken up through the apoB/E receptor pathway and induce a membrane 'blebbing' and a marked loss of cell viability on lymphoid cells [14]. Similar morphological alterations have been shown to be caused by toxic compounds generating a cellular oxidative stress and a sustained rise of the cytosolic free calcium concentration, $[Ca^{2+}]_i$ [17–21].

This prompted us to investigate the effect of oxidized LDL on calcium homeostasis and on cell viability of bovine aortic endothelial cells (BAEC) which are potentially one of the primary targets of oxidized LDL [22]. We report in this paper that relatively low doses of oxidized LDL ('pulse-chase' experiments) elicit a dramatic slow and sustained rise of the $[Ca^{2+}]_i$ concomitant with a drop of cytosolic pH, without affecting the viability of BAEC. Higher doses of oxidized LDL (continuous 'pulse' experiments) generate similarly a $[Ca^{2+}]_i$ rise associated, in this case, with morphological alterations of BAEC and followed by a loss of cell viability. The possible relationship between the sustained rise of cytosolic calcium induced by oxidized LDL and the sequence of cellular events leading to pathological events (the most stricking being cell death and defect of endothelial cell lining) is discussed.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Calcium (Quin-2/AM and Fluo3/AM) or fluorescent pH (BCECF/AM) probes and nigericin were obtained from Molecular Probes (Eugene, OR, USA), ^{51}Cr (300 mCi/mmol) from Amersham (Paris, France), MTT, bovine serum albumin from Sigma (St. Louis, MO, USA), RPMI 1640 and Phenol red free- (PRF-) RPMI 1640, fetal calf serum, glutamine, penicillin and streptomycin from Gibco (Cergy-Pontoise, France) and Ultroser G from IBF (Villeneuve-la-Garenne, France). Other reagents and chemicals were obtained from Merck (Darmstadt, Germany) or Prolabo (Paris, France).

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2.2. *Culture of bovine aortic endothelial cells (BAEC)*

The bovine aortic endothelial cell line (GM 7372A) was obtained from the NIGM human genetic mutant cell repository (Camden, NJ, USA) and was grown in RPMI 1640 medium containing 10% fetal calf serum, penicillin, streptomycin and glutamine. 48 h before LDL incorporatation, this medium was removed and replaced by RPMI 1640 containing 2% Ultroser G (a serum substitute devoid of lipoprotein), as previously used [16].

2.3. *LDL isolation and oxidation*

LDL were isolated from human pooled fresh sera by ultracentrifugation according to Havel [23], dialyzed, sterilized by filtration and their purity controlled as previously indicated [15,16]. Purified LDL were stored at 4°C under nitrogen (up to 2 weeks).

For each experiment, 2–4 mg (as apoB) of LDL were exposed to UV-C radiations (254 nm, 0.5 mW/cm^2, for 2 h, under the standard conditions) and immediately incorporated (at the concentrations indicated in the text) in the culture medium [16].

2.4. *Determination of cytotoxicity*

The cytotoxicity was determined under the previously used conditions [16], with slight modifications. LDL (200 μg apoB/ml), sterilized by filtration on 0.2 μm Millipore membrane, were added to the culture medium. Two alternative procedures have been used: (i) in continuous pulse experiments, cells were kept in contact with LDL until the cell viability was determined (at the end of the 48 h-pulse period, under the standard conditions); (ii) in pulse-chase experiments, cells were in contact with LDL for 5 h, then the medium was discarded and replaced by a lipoprotein-free medium, until the cell viability was determined (up to 48 h when indicated). The cell viability was determined by the MTT test [25].

In order to determine ^{51}Cr release, BAEC were incubated in RPMI medium containing 2% Ultroser G and ^{51}Cr (10^7 dpm/5 ml) at the end of the pulse, then (at the required time) cells were washed twice with fresh medium and the ^{51}Cr released in the culture medium after 1 h incubation was counted [26].

2.5. *Determination of $[Ca^{2+}]_i$ and pH_i*

The concentration of $[Ca^{2+}]_i$ (free cytosolic calcium concentration) was determined by using Quin-2/AM (the permeant non-fluorescent Quin-2/AM being hydrolyzed by intracellular carboxylesterases to liberate the intracellular trappable fluorescent calcium indicator Quin-2). Briefly, BAEC were incubated during the 'chase' in 25 mmol/l HEPES-buffered RPMI 1640 medium containing BSA 0.5% and Quin-2/AM (5 μmol/l) until $[Ca^{2+}]_i$ determination. At the required time, after washing with RPMI 1640 and twice with phosphate-buffered saline buffered with HEPES (25 mmol/ l), cells were immediately used to determine the fluorescence F and the calibration of the dye response (by measuring F_{min} and F_{max}) exactly as described by Arslan et al. [27]. For fluorescence microscopy studies, Fluo-3/AM [28,29] was used as indicated by the manufacturer (2 μmol/l in 0.5% DMSO and 0.02% Pluronic F-127 [28]).

Intracellular pH (pH_i) was determined by using BCECF/AM (2 μmol/l, solubilized in 1% DMSO) under the conditions indicated by the manufacturer [28], using a calibration of the pH-dependent fluorescence with nigericin according to the method of Thomas et al. [30].

2.6. *Determination of thiobarbituric acid reactive substances (TBARS) and proteins*

Lipid peroxidation was evaluated by determining the content of thiobarbituric acid reactive substances (TBARS) according to the method of Yagi [31] as previously used [15].

Protein concentrations were determined using the procedure of Lowry et al. [32].

2.7. *Electron microscopy*

BAEC were fixed in a 2.5% glutaraldehyde in 0.4 M pH 7.4 cacodylate-HCl buffer at room temperature, dehydrated by ethanol and isoamyl acetate, then gold-coated and examined by electron scanning microscopy using a Jeol 25 microscope [14,16].

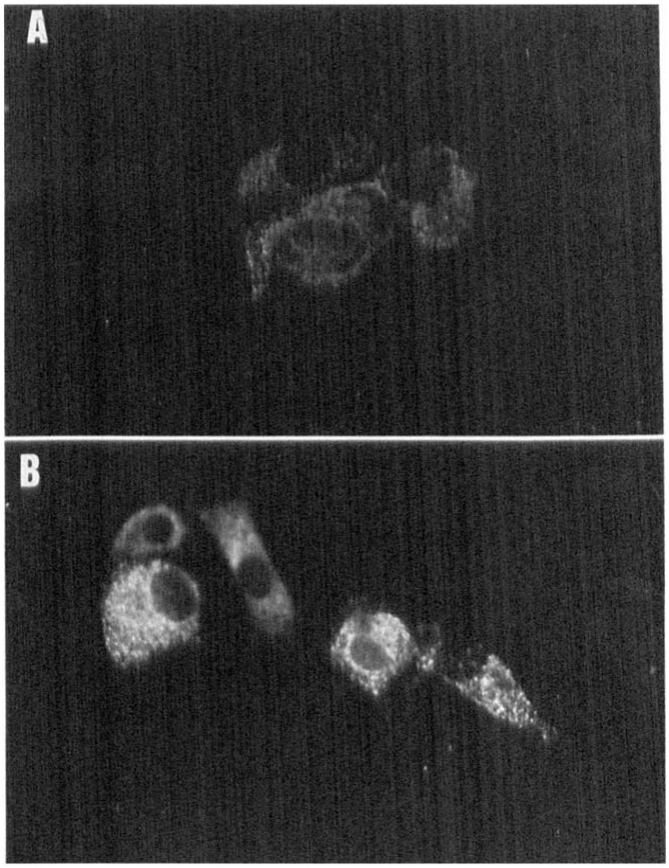
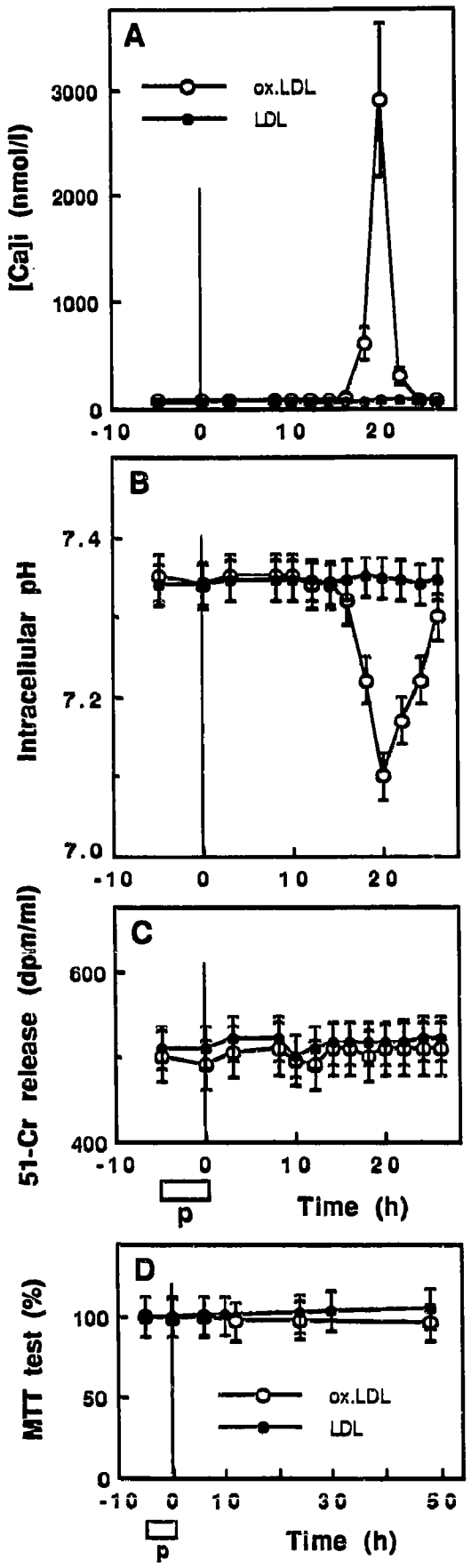


Fig. 1. Comparative time course of $[Ca^{2+}]_i$ (A), intracellular pH (pH_i) (B) and cytotoxicity indexes, i.e. ^{51}Cr release (C) and MTT test (D) in BAEC submitted to a 5 h-pulse (white bar marked with 'p') with a fixed concentration (200 μg apoB/ml) of oxidized LDL (oxLDL) or non-oxidized LDL (LDL). Then, cells were washed twice with RPMI medium and grown in RPMI medium containing 2% Ultroser G. The $[Ca^{2+}]_i$ concentration (evaluated using Quin-2/AM) and pH_i (evaluated using BCECF/AM) were determined under the conditions indicated in Materials and Methods. Experiments were done in quadruplicate.

3. RESULTS

3.1. *Rise of $[Ca^{2+}]_i$ and drop of pH_i in short pulse with oxidized LDL (Figs. 1 and 2)*

As shown in Fig. 1A, the basal level of $[Ca^{2+}]_i$ was around (70±10 nmol/l) in non-stimulated BAEC. When BAEC were pulsed for 5 h with oxidized LDL (200 μg apoB/ml), a major rise of the cytosolic calcium concentration was observed several hours after the end of the pulse period: under the experimental conditions used, $[Ca^{2+}]_i$ began to rise at 18 h and reached the maximum (estimated around 2800 nmol/l) 2 h later and then decreased back to the basal level in around 2 h. In the same time, we observed a significant drop of pH_i (0.25±0.05 pH units under the basal pH_i level, 7.35) (Fig. 1B). In comparison, the pH_i of BAEC pulsed with



(non-oxidized) LDL was not significantly modified over the period of time of observation. During all this period the release of ^{51}Cr (Fig. 1C) was not altered suggesting that the increase of $[Ca^{2+}]_i$ did not result from a non-specific alteration of the plasma membrane permeability to ions. The integrity of the plasma membrane barrier was confirmed by the MTT test which was not significantly modified (Fig. 1D) over a period of 48 h. Note that the $[Ca^{2+}]_i$ in cells pulsed with non-oxidized LDL did not significantly change over the observation time period (i.e. during 24 h after the end of the pulse with LDL).

As shown in Fig. 2, the sustained rise of $[Ca^{2+}]_i$ induced by oxidized LDL in BAEC loaded by Fluo-3/AM was clearly visualized by fluorescence microscopy, whereas BAEC pulsed with (non-oxidized) LDL did not differ from the control (without LDL).

3.2. *Rise of $[Ca^{2+}]_i$ and cytotoxicity in continuous pulse by oxidized LDL (Figs. 3 and 4)*

When BAEC were pulsed continuously with relatively high levels of oxidized LDL (200 μg apoB/ml), the peaks of $[Ca^{2+}]_i$ and pH_i were earlier and broader and we observed a cytotoxic effect as shown by the MTT test (Fig. 3): under these experimental conditions, the cytotoxicity began to rise just after the maximum of the peak of $[Ca^{2+}]_i$ and increased during the next 48h.

Under these conditions, as shown by scanning electron microscopy, BAEC exhibited morphological changes of the plasma membrane such as blebbing (Fig. 4B) followed by more severe alterations such as disruptions, holes and tears of the plasma membrane (Fig. 4C) which are associated with a marked loss of viability.

4. DISCUSSION

The most prominent result of this paper is the delayed sustained rise of $[Ca^{2+}]_i$ (more than 20–40 times the basal level) elicited in cultured BAEC by relatively low doses (around 10 pmol apoB/mg cell protein in 5 h-pulse) of oxidized LDL, without apparent loss of cell viability. The very long lag period (between uptake of oxidized LDL and $[Ca^{2+}]_i$ rise) could result from a sequence of intermediate intracellular steps: uptake and routing of oxidized LDL to lysosomes [16], lipid degradation by lysosomal hydrolases (data not shown), release and transport of oxidation derivatives from the

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Fig. 2. $[Ca^{2+}]_i$ imaging by Fluo-3/AM fluorescence microphotography of BAEC treated by the 5 h-pulse procedure under the experimental conditions of Fig. 1. Cells, grown on cover slides, were loaded with 2 μmol/l Fluo-3/AM at the end of the pulse and were examined repetitively by fluorescence microscopy. (A) Resting BAEC (cultured with non-oxidized LDL). (B) BAEC pulsed for 5 h with oxidized LDL (200 μg apoB/ml) and examined at the peak of $[Ca^{2+}]_i$ (20 h after the end of the pulse).

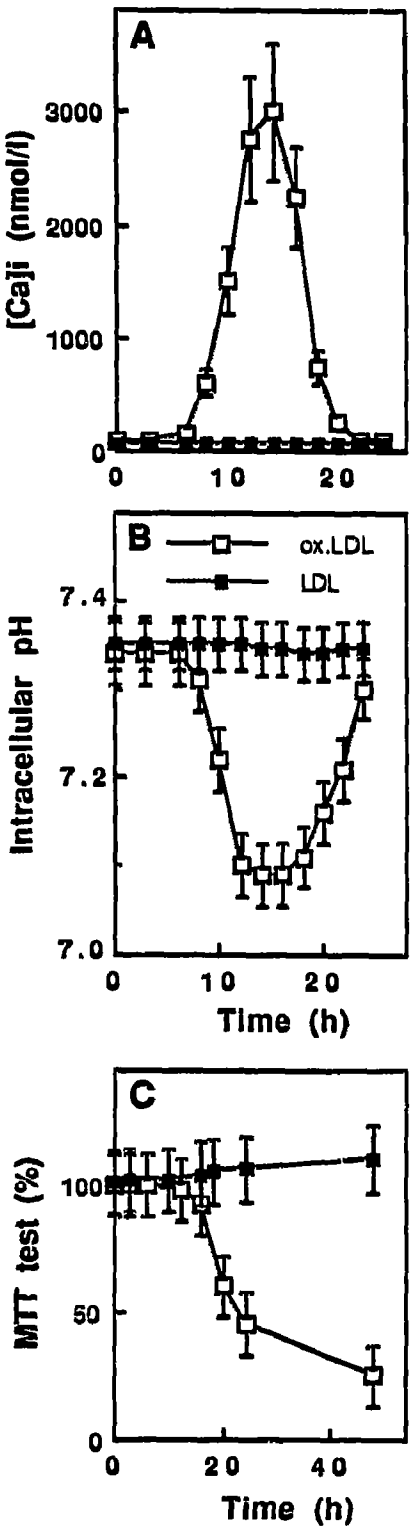

Fig. 3. Time courses of $[Ca^{2+}]_i$ (A), pH_i (B) and cytotoxicity (C) in BAEC pulsed continuously with oxidized LDL (oxLDL) or non-oxidized LDL (LDL). Cells were grown in RPMI medium containing 2% Ultroser G and a fixed concentration of oxidized LDL (200 μg apoB/ml). $[Ca^{2+}]_i$ concentration, pH_i and cytotoxicity were evaluated as indicated in Fig. 1. Experiments were done in triplicate.

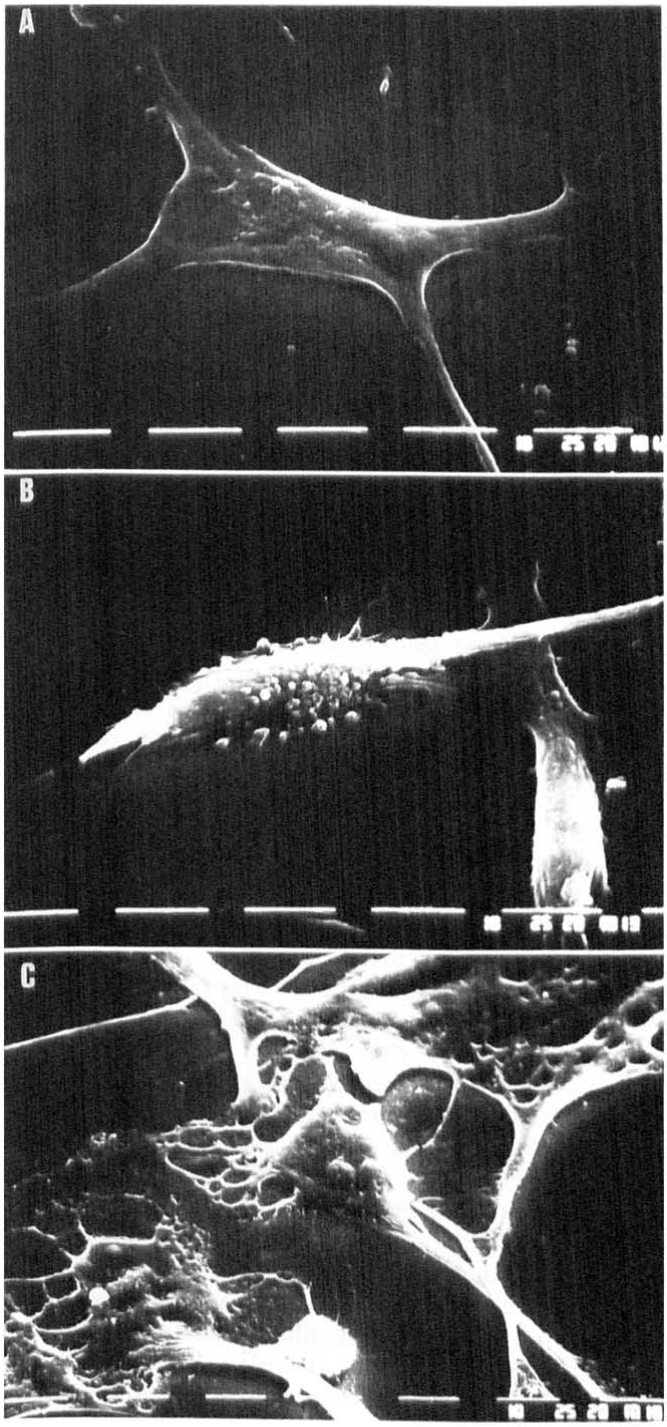

Fig. 4. Scanning electron microphotography of BAEC grown in the standard RPMI medium containing 100 μg/ml LDL (A) or oxidized LDL (B and C). Cells were examined with a Jeol 25 electron scanning microscope under the conditions previously used [16]. (A) Normal flat-spread BAEC with regular cytoplasmic surface (× 1,500). (B and C) BAEC were cultured continuously with 200 μg/ml oxidized LDL; at time 18 h, BAEC were spindle-shaped, retracted and exhibited plasma membrane blebbing (B) (× 1,500); at time 48 h, BAEC exhibited holes and disruptions of the plasma membrane (C) (× 1,000), white bars represent 10 μm.

lysosomes towards the cytoplasmic targets and possible involvement of a metabolic process (intermediary signalling metabolites?) leading to the cytoplasmic influx of Ca^{2+} from extracellular medium or from intracellular stores. From the lack of ^{51}Cr leakage, we can conclude that the plasma membrane is not directly attacked by peroxides or other oxidation derivatives (contained in oxidized LDL) which have been reported to induce alterations of the permeability (natural or synthetic) of biomembranes [33–35]. Thus, all these data rather suggest the existence of a transduction mechanism (yet completely unknown) between signal molecules brought or generated by oxidized LDL and the $[Ca^{2+}]_i$ rise. It is noteworthy that the timing (delayed, slow and sustained rise) and intensity (very high peak) of the $[Ca^{2+}]_i$ signal are completely different from the usually rapid response to Ca^{2+}-mobilizing agonists [36] or to non-oxidized LDL [37]. On the other hand, the regulation systems of the $[Ca^{2+}]_i$ (i.e. extrusion of cytosolic Ca^{2+} towards outside the cell or towards the intracellular stores) seem to be preserved since after the sustained $[Ca^{2+}]_i$ rise, the level of $[Ca^{2+}]_i$ slowly decreased in several hours (by a as yet unknown mechanism). The intracellular acidification could be the consequence of the sustained $[Ca^{2+}]_i$ rise which leads to H^+ metabolic generation as previously demonstrated in T cells [38].

Morphological changes shown by electron scanning microscopy of BAEC cultured in the presence of oxidized LDL are consistent with the observed $[Ca^{2+}]_i$ rise which is known to induce cell contraction [39,40] and regulation of endothelial barrier permeability [41]. As oxidized LDL have been detected in atherosclerotic lesions [42], it is not excluded that similar phenomena could occur in vivo, thus raising in the underlying subendothelial space the concentration of LDL and other plasma proteins which are thought to play a role in atherogenesis [43,44].

Under conditions inducing a cytotoxic effect (higher doses of oxidized LDL internalized by cells, as shown previously with lymphoid cells) [16], the $[Ca^{2+}]_i$ peak was higher and more sustained than in 5 h-pulse experiments. The morphological alterations observed here (i.e. blebbing and loss of the plasma membrane integrity) observed in electron microscopy and demonstrated by the leakage of cytoplasmic enzymes could result from the sustained $[Ca^{2+}]_i$ rise and from the subsequent activation of cytoplasmic calcium-dependent enzymes, such as proteases able to break proteins anchoring actin microfilaments to the plasma membrane [45] or phospholipases A2 (additionally activated by the presence of oxidized fatty acids in phospholipids) able to generate large amounts of lysophospholipids which can in turn destabilize the lipid bilayer [46]. Although it is not clear whether the uncontrolled $[Ca^{2+}]_i$ rise is a prerequisite to cell death or whether it accelerates the formation of necrotic lesions only after the occurrence of lethal lesions (possibly independent of $[Ca^{2+}]_i$; it is likely that the uncontrolled $[Ca^{2+}]_i$ rise is involved in the genesis of morphological changes characteristic of injured cells [20,45].

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